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# to Meiotic Chromosomes of Spermatocytes as They Initiate Meiosis

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**Meiosis, the fundamental evolutionarily conserved differentiative process by which haploid gametes are produced, is a complex and tightly regulated nuclear process. The murine *Meig1* gene was previously shown to have a germ cell-specific transcript which is abundantly expressed during meiosis, in both males and females, suggesting that it is involved in meiotic processes. Protein analysis revealed that MEIG1 appears in multiple phosphorylated forms, including two dimeric forms of $M_r$ 31000 and 32000, which exhibit a developmentally regulated switch in their relative abundance. The tyrosine-phosphorylated $M_r$ 31000 form becomes the dominant form once the cells enter meiosis. In this study we show that the $M_r$ 31000 dimeric form appears in the nuclear fraction of testicular protein extract, whereas the $M_r$ 32000 dimeric form and the monomeric forms of MEIG1 remain cytoplasmic. The appearance in the nuclear fraction is developmentally regulated, coinciding with progression of the first spermatogenic wave through meiotic prophase I. Utilizing immunocytochemistry we show that nuclear localization is apparent in primary spermatocytes through their maturation into elongated spermatozoa, but not in either somatic cells or germ cells from early postnatal pups. We also show that MEIG1 associates specifically with meiotic chromosomes *in vivo*. These results indicate that in germ cells, the $M_r$ 31000 dimeric form enters the nucleus during the first meiotic prophase and binds to the meiotic chromatin. Possible nuclear functions, as well as possible modes of nuclear localization, are discussed.** © 1999 Academic Press

***Key Words:*** **mouse; spermatogenesis; meiosis; MEIG1 (Meg1); nuclear localization; chromatin binding.**

## INTRODUCTION

The essence of gamete production involves reducing the number of chromosomes in the gametes by half so that following the fusion of sperm and egg, a new diploid organism is formed. This reduction in chromosome number is achieved by two successive cell divisions without an intervening DNA replication step, a process that is known as meiosis. Due to a lack of an efficient *in vitro* system for germ cell differentiation, our understanding of the molecular regulatory mechanisms involved in specific meiotic events in mammals has been very limited. In recent years, an increasing number of genes that are expressed during mammalian spermatogenesis and might have a regulatory role have been identified. Some of these genes encode protein kinases, widely accepted mediators of molecular regulatory mechanisms. These include, among others, *Mak* (Matsushime *et al.*, 1990; Jinno *et al.*, 1993), *Gek1* (Yanagisawa *et al.*, 1996), *Nek2* (Rhee and Wolgemuth, 1997; Arama *et al.*, 1998), *Ayk1* (Yanai *et al.*, 1997), *hPim2* (Baytel *et al.*, 1998), *Tlk* (Shalom and Don, 1999), and some forms of cyclin-dependent kinases (Rhee and Wolgemuth, 1995; Ravnik and Wolgemuth, 1996), as well as kinases that are expressed during later stages of spermatogenesis, in haploid spermatids, such as the cellular proto-oncogenes *c-mos* and *Pim1* (Sorrentino *et al.*, 1988; Iwaoki *et al.*, 1993). Other spermatogenesis-expressed genes with potential regulatory roles are the zinc-finger-containing genes *Zfp-35*, *Zfp-38*, and *Zfp-51* (Cunliffe *et al.*, 1990; Chowdhury, 1992; Burke *et al.*, 1994) and the neuropeptide-coding genes *POMC* and proenkephalin (Gizang-Ginsberg and Wolgemuth, 1987; Mehta *et al.*, 1994). Nevertheless, most of these kinases have not been assigned specific substrates and only few gene products have been assigned a specific spermatogenic

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regulatory function. One such example is the transcriptional activator CREM (cyclic AMP-responsive element modulators) and *cis* elements that are required for its activity to confer postmeiotic haploid gene expression in mice (Sun and Means, 1995; Nantel *et al.*, 1996; Blendy *et al.*, 1996).

*Meig1* (formally designated *meg1;* Don and Wolgemuth, 1992) is a murine gene that encodes two alternatively spliced transcripts which are differentially expressed in testicular somatic cells, most abundantly in Leydig cells, and in germ cells (Ever *et al.*, 1999). The germ-cell-specific transcripts (0.75 kb in size) begin to accumulate in early stages of the first meiotic prophase, leptotene–zygotene, and are most abundant in pachytene spermatocytes. No *Meig1* transcripts could be detected in ovaries of adult females. However, this transcript could be detected in ovaries of embryos at days 16.5–17.5 of gestation, when the oocytes have reached a comparable meiotic stage, i.e., the pachytene stage of prophase I (Don and Wolgemuth, 1992; Don *et al.*, 1994). This suggests that *Meig1* is involved in meiosis- rather than in spermatogenesis-specific processes. Moreover, protein analysis revealed that the MEIG1 protein appears in multiple phosphorylated forms including two dimeric forms of $M_r$ 31000 and 32000. A developmentally regulated switch in the relative abundance of these two dimeric forms is apparent, in which the $M_r$ 31000 form, which is tyrosine phosphorylated, becomes dominant once the cells enter meiosis (Chen-Moses *et al.*, 1997). The involvement of MEIG1 in meiotic events thus appears to be dependent on dimerization and phosphorylation/dephosphorylation reactions.

In this study we demonstrate that the $M_r$ 31000 dimeric form of MEIG1 enters the nucleus of primary spermatocytes as they initiate meiosis and binds to the meiotic chromosomes. We suggest that MEIG1 might play a role either in the regulation of meiotic events or in meiotic chromatin organization.

## MATERIALS AND METHODS

### *Source of Tissues*

Balb/c mice, >60 days of age, were used as a source of normal mouse tissues for all experiments unless otherwise stated. Tissues for protein extraction were frozen in liquid nitrogen immediately upon dissection and stored at −70°C until used. All animals were sacrificed by cervical dislocation.

### *Protein Extraction*

Nuclear as well as cytoplasmic proteins were extracted according to Lee *et al.* (1998). In brief, testes (or other tissues) from mature male mice and from pups at different developmental stages were washed in cold PBS after dissection, homogenized in 1 tissue volume of buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, and 1 mM $Na_3VO_4$), and incubated on ice for 15 min. Cells were lysed by forcing the suspension ~10 times through a 25-gauge hypodermic needle. Soluble cytoplasmic proteins were obtained by centrifugation of lysates at 12,000*g* for 20 s and collecting the supernatant. For nuclear proteins, the crude pellet was resuspended in 1/3 original tissue volume of buffer B (20 mM Hepes, pH 7.9, 25% glycerol, 15 mM $MgCl_2$, 0.01 M KCl, 0.5 mM EDTA, 0.5 mM PMSF, and 1 mM $Na_3VO_4$) to which an equal volume (1/3 original tissue volume) of buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 15 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM PMSF, and 1 mM $Na_3VO_4$) was gradually added, with stirring on ice for 30 min. The suspension was centrifuged at 12,000*g* for 5 min, and the supernatant (which contained the nuclear proteins) was removed and dialyzed against 1 liter of buffer D (20 mM Hepes, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 1 mM $Na_3VO_4$) for 2 h. The pellet was resuspended in a small volume of SDS (2%)-containing buffer D and was sonicated at maximum intensity for 30 s. This was regarded as cytoplasmic pellet or insoluble cytoplasmic fraction. A cocktail of protease inhibitors (complete protease inhibitor cocktail tablets; Boehringer Mannheim) was included in all buffers according to the manufacturer's recommendations.

### *Immunoblotting (Western) Analysis of Proteins*

Protein extracts were separated on a 15% SDS–polyacrylamide gel and electroblotted onto a nitrocellulose filter (using the Mini-Protein II cell and the MiniProtein II transfer cell (Bio-Rad), respectively) according to standard protocols. After electrotransfer, filters were washed in water for 3 min, monitored by staining with Ponceau stain (Sigma), and then blocked with 1% BSA in TBST (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. Affinity-purified rabbit anti-MEIG1 polyclonal antibodies (Chen-Moses *et al.*, 1997) were used as primary antibodies at a dilution of 1:20,000. Unbound antibodies were removed by three washes in TBST. Anti-rabbit IgG-AP (alkaline phosphatase conjugated) antibodies (Promega) were used as secondary antibodies at dilutions of 1:7500. Binding was detected by incubation with alkaline phosphatase substrate (Promega) for 15–30 min followed by washing of the unused substrate with water.

### *Preparation of Testicular Cell Suspension*

Testes were dissected into a petri dish containing ice-cold sterile separation medium (4 mM L-glutamine, 1.5 mM sodium pyruvate, 10% fetal calf serum, and 75 $\mu$g/ml ampicillin in Dulbecco's modified eagle medium containing nonessential amino acids). For each experiment with a given developmental stage, testes from several animals (six to eight pups in early postnatal animals or two mature animals) were pooled and processed. Each testis was decapsulated by making a small incision in the testis and forcing (using sterile tweezers) the contents of the testis through the incision into a 15-ml Falcon tube containing 5 ml ice-cold separation medium. Next, 0.25 ml collagenase (Calbiochem) from a 2 mg/ml stock solution (prepared in separation medium) was added to the tube with the decapsulated testes and incubated for 5 min at 35–37°C under vigorous shaking. The seminiferous cords were allowed to sediment to the bottom of the tube while the suspension was incubated on ice. The seminiferous cords were washed twice in 10 ml separation medium, resuspended in 12 ml separation medium containing 1 unit/ml DNase I (Boehringer Mannheim), incubated 2 min in 35–37°C, and transferred to ice. Using a Pasteur pipette, the seminiferous cords were teased into a single cell

suspension which was then filtered through a 50-$\mu$m nylon mesh and washed twice with separation medium (centrifugation at 200–300*g*).

### *Immunocytochemical Analysis*

Testicular cells (see previous section) attached to poly-L-lysine-coated slides, were fixed in 4% paraformaldehyde (in PBS) for 30 min at room temperature, washed three times in PBS, and incubated in blocking solution (20% goat normal serum, 0.1% Triton X-100 in PBS) for 1 h at room temperature. Primary antibody (rabbit anti-MEIG1), diluted 1:1000 in blocking solution, was applied to the cells at room temperature for 1 h (alternatively, overnight at 4°C) and then removed by three washes in PBST, 15 min each. FITC (fluorescein isothiocyanate)-conjugated goat anti-rabbit IgG antibody (Zymed), diluted 1:50 in PBS containing 20% goat serum, was used as a secondary antibody (1 h incubation at room temperature, in the dark), followed by two washes in PBST (10 min each), 5 min staining of nuclei with PI solution (10 mM Tris, pH 8.0, 1 mM NaCl, 0.1% Nonidet P-40, 0.7 mg/ml RNase A, 0.05 mg/ml propidium iodide), and two washes in PBST (15 min each) to remove excess dye. One drop of antibleaching retardant (Bio-Rad) was added to the cells before sealing with a coverslip. Cells were viewed with the MRC 1024 confocal microscope (Bio-Rad) using an argon/krypton laser. Excitation was at 494 nm for FITC and at 536 nm for PI.

### *FACS Analysis and Chromosome Preparation*

Testicular cell suspensions from pups at different postnatal developmental stages, as well as from mature males, were brought to a concentration of $2 \times 10^6$ cells/ml in separation medium and diluted in a 1:1 ratio with propidium iodide solution. Cells were analyzed by a Becton–Dickinson FACStar Plus instrument (fluorescence-activated cell sorter), equipped with an argon laser, within 2 h of staining. Excitation was at 488 nm and emission at 585 nm. The various cell populations (containing 4D, 2D, or 1D DNA) were sorted according to Malkov *et al.* (1998) into a tube containing 1 ml separation medium. The purity of the sorted cell populations was about 98%, as determined by reanalyzing the sorted cell populations by the FACS. For nuclear or chromosome preparations, the various purified cell populations were washed three times in PBS and resuspended in hypotonic solution (0.075 M KCl) for 20 min at 37°C. Cells were fixed by adding several drops of fixative (methanol and acetic acid in a ratio of 3:1) to the hypotonic solution (1–2 min), washing once with fresh fixative, and incubating in fresh fixative for at least 30 min at 4°C. Cells were then centrifuged, the fixative was removed, and the cells were resuspended in residual fixative and dropped onto Tespa (aminopropyltriethoxysilane; Sigma)-treated slides. Chromosomes (or nuclei) were stained as described above for immunocytochemistry; however, the blocking solution did not contain Triton X-100. For mitotic chromosomes, lymphocytes (2B4 cell line) were grown in cell culture for 72 h (subconfluent culture). Twenty-four hours after replacing the medium, colchicine (Sigma) was added to a concentration of 5 $\mu$g/ml overnight, after which the medium was replaced by hypotonic solution. All the following steps were performed as described above for spermatocytes.

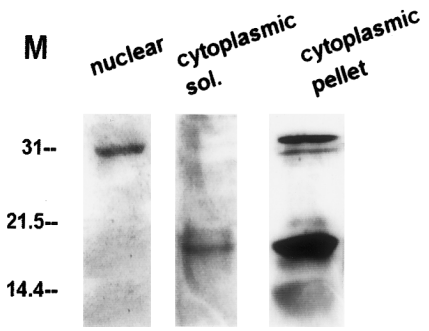


**FIG. 1.** Western analysis of nuclear and cytoplasmic proteins extracted from testes of normal mature mice. The cytoplasmic proteins were divided into a soluble (sol.) and insoluble (pellet) fraction. Affinity-purified rabbit anti-MEIG1 antibodies and AP-conjugated anti-rabbit IgG-antibodies were used to detect MEIG1 protein. Size markers ($M_r$, $\times$1000) are indicated at the left.

## RESULTS

### *The $M_r$ 31000 Dimeric Form of MEIG1 Localizes to the Nucleus*

MEIG1 is a small basic protein (p*I* 9.16), a property compatible with its being a nuclear protein. This assumption has been supported by predicting, with a certainty of 60%, that MEIG1 is a nuclear protein, using the PSORT software (Nakai and Kanehisa, 1992). To test this prediction, cytoplasmic and nuclear protein extracts from testes of mature mice were analyzed by Western blotting using highly specific affinity-purified rabbit anti-MEIG1 antibodies (Chen-Moses *et al.*, 1997). Only the $M_r$ 31000 dimeric form of MEIG1 was detected in the nuclear extracts, whereas the monomeric forms and the $M_r$ 32000 dimeric form were detected in the cytoplasmic extracts, with low levels in the soluble cytoplasmic fraction and much higher levels in the insoluble cytoplasmic fraction (Fig. 1). Residual amounts of the $M_r$ 31000 form were detected in the cytoplasmic extracts (Fig. 1). These results validated the prediction that at least one of the MEIG1 forms, the $M_r$ 31000 dimeric form, localizes to the nucleus of testicular cells, although not specifying the exact cell population. These results also suggest that in the cytoplasm, MEIG1 does not appear as a soluble protein but rather in an insoluble form, perhaps within special vesicles or other organelles. Immunoelectron microscopy experiments are currently being performed to address the cytoplasmic localization of MEIG1.

### *MEIG1 Localizes to Nuclei of Primary Spermatocytes*

In which testicular cells does MEIG1 localize to the nucleus? Does the nuclear localization of MEIG1 correlate

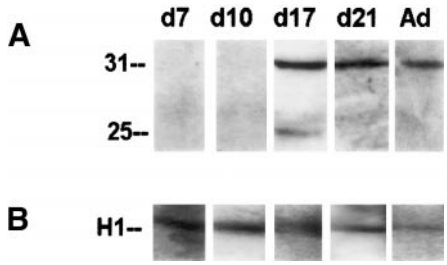


**FIG. 2.** Western analysis of nuclear proteins extracted from testes of animals at different postnatal developmental stages. Each lane on the filter, onto which the different nuclear proteins were blotted, was divided into two identical halves. One half was analyzed by anti-MEIG1 antibodies (A), while the other half was analyzed by monoclonal anti-histone H1 antibodies (B), to verify nuclear origin and protein quality. Size markers ($M_r$, $\times 1000$) are indicated at the left of A.

with the developmentally regulated dominance of the $M_r$ 31000 dimeric form? To address these questions we analyzed, by Western blotting, nuclear proteins extracted from testes of animals at different postnatal (pn) developmental stages. A detailed differentiation schedule of the first spermatogenic wave in mice has been described (Bellve *et al.*, 1977a,b), enabling us to determine the spermatogenic stage at which a particular biochemical or cellular event occurs. At pn day (d) 6 the seminiferous epithelium of the testis contains only primitive type A spermatogonia and Sertoli cells. At pn d8, type A and type B spermatogonia are present, and by pn d10, cells from the first spermatogenic wave can be found at the preleptotene and leptotene stages of the first meiotic prophase. Zygotene primary spermatocytes are first detected on pn d12, and early pachytene and late pachytene spermatocytes first appear on pn d14–15 and 18–21, respectively. Haploid round spermatids first appear at about pn d21, and at approximately pn d30, the complete spermatogenic complement is present in the seminiferous epithelium. In this set of experiments, a Ponceau-stained filter, onto which nuclear proteins from testes of pups at different developmental stages were transferred, was cut into strips so that each lane was divided into two identical halves. One-half of each lane was analyzed with anti-MEIG1, while the other half was analyzed with anti-histone H1 monoclonal antibody (NeoMarkers, Fremont, CA) as a control for the nuclear origin of the analyzed proteins. As can be seen in Fig. 2, no signal corresponding to the $M_r$ 31000 form, or to any other MEIG1 form, was seen in nuclear extracts from early postnatal testes (days 7 and 10), whereas the histone H1 signal was readily detected, indicating the existence of nuclear proteins. At these early postnatal developmental stages the majority of the cells in the testis are somatic cells, mainly Sertoli and Leydig cells (Bellve *et al.*, 1977a). It thus seems clear that these cells do not contain detectable amounts of MEIG1 in their nuclear fraction. However, as cells from the first spermatogenic wave enter meiosis, the $M_r$ 31000 form starts to appear in the nuclear extracts, and by pn d17 and later the $M_r$ 31000 signal is obvious (Fig. 2). These results suggest that the $M_r$ 31000 nuclear form of MEIG1 is restricted to germ cells at prophase I, and probably at later developmental stages as well, and point at a correlation between the developmentally regulated dominance of the $M_r$ 31000 form and its nuclear localization. Interestingly, when protein extracts from various somatic tissues (brain, heart, liver, kidney) were analyzed using the anti-MEIG1 antibodies, the nuclear form of MEIG1 could not be detected (not shown), supporting the notion that the nuclear localization of the $M_r$ 31000 form is characteristic of germ cells. The fainter signal obtained with the histone H1 antibody, when proteins from a more advanced developmental stage were analyzed, seems to reflect the replacement of histones by protamines in differentiated germ cells, together with the increased percentage of these germ cells among all testicular cells. A faint $M_r$ 25000 band of an unknown nature could occasionally be detected in the nuclear extracts of the later developmental stages as well.

To complement these results, immunocytochemical localization experiments for the MEIG1 protein were performed with testicular cell suspensions originating from testes of pups at different postnatal ages. When cells obtained from testes of early postnatal pups (day 7–8) were analyzed, the MEIG1 signal could be detected in the cytoplasm of the cells but not in the nucleus (not shown). These positively stained cells might include premeiotic spermatogonia cells and/or testicular somatic cells, among which Sertoli and Leydig cells are most abundant. Furthermore, a very similar pattern of staining was obtained when 2D cells (diploid cells at G1 of the cell cycle) that were purified by FACS sorting from a total population of normal mature testicular cells were analyzed (Fig. 3d). Since this 2D population represents a mixture of premeiotic germ cells and somatic cells, it appears that this pattern of staining (i.e., cytoplasmic but not nuclear) is indeed characteristic of the premeiotic germ cells and the somatic cells, which is in agreement with the results shown in Fig. 2. As cells progressed to prophase I, MEIG1 started to appear in the nucleus of cells, and by the time primary spermatocytes from the first spermatogenic wave reached the pachytene stage (pn d14–17), nuclear signal was apparent in about 40% of the cells (Fig. 3a). Other cells showed cytoplasmic signal only, or no signal at all. In postmeiotic haploid round spermatids (pn d21), considerable amounts of MEIG1 could be detected in the nuclei (Fig. 3b), and in elongated spermatozoa (pn d30), the chromatin was heavily stained by the anti-MEIG1 antibodies (Fig. 3c). It should be emphasized that the nuclear localization of MEIG1 was verified by optical sectioning through the nuclei of the cells using confocal microscopy. No signal corresponding to MEIG1 could be detected in control experiments with secondary

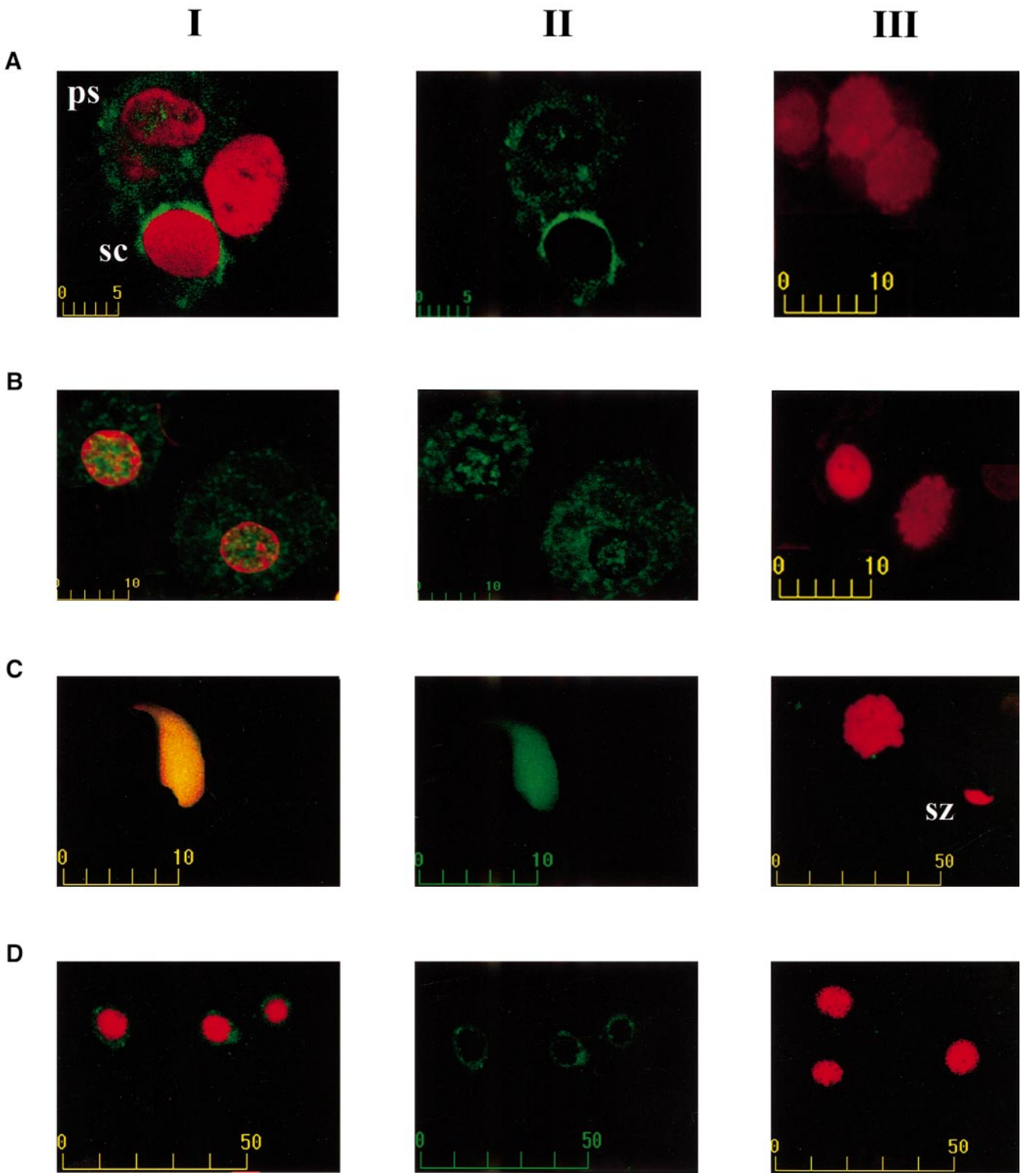


**FIG. 3.** Immunocytochemical localization of the MEIG1 protein in testicular cell suspensions from normal mice at different developmental stages. Affinity-purified rabbit anti-MEIG1 antibodies were used as primary antibodies, and staining was visualized with

antibody alone (FITC-conjugated goat anti rabbit IgG), with preimmune serum, or with anti-MEIG1 antibodies preadsorbed with recombinant MEIG1 prior to use (Figs. 3a–3d). These results suggest that the $M_r$ 31000 form of MEIG1 enters the nucleus of the primary spermatocytes as they initiate and progress through meiosis and that the nuclear localization persists developmentally through to the stage of elongated spermatozoa.

### *MEIG1 Interacts Directly with Meiotic but Not Mitotic Chromosomes*

The localization of MEIG1 to nuclei of germ cells from primary spermatocytes through their maturation to elongated spermatozoa suggested that MEIG1 might directly interact with the cell chromatin. To first test the potential DNA binding properties of MEIG1, we performed Southwestern analyses in which *Eco*RI-digested native mouse genomic DNA was probed with either recombinant MEIG1 or various testicular protein extracts. Using anti-MEIG1 and the ECL detection system (Amersham), we were able to demonstrate a strong signal along the digested DNA when it was probed with nondenatured testicular nuclear fraction or with the recombinant MEIG1 protein (not shown). Probing with a nondenatured cytoplasmic fraction or with SDS-denatured extracts from total testis did not show significant binding. Nonetheless, the ability of the SDS-denatured proteins to bind DNA was restored by dialysis of the SDS extracts in PBS for a week (not shown). No signal was obtained in control experiments in which DNA was probed with fetal calf serum or when preimmune serum or preadsorbed MEIG1 antibodies were used for detection. These results indicated that MEIG1 can bind DNA as a native protein, at least *in vitro*. However, the smeary signal obtained with digested genomic DNA could as well reflect an *in vitro* artifact in which a basic protein binds DNA nonspecifically, with no implications for the cell *in vivo*. To test whether MEIG1 in fact interacts *in vivo* with the meiotic chromatin, we performed *in situ* analysis on nuclei and meiotic chromosomes, prepared from FACS-purified specific testicular cell populations (Malkov *et al.*, 1998). In all cases detection of MEIG1 attachment to the nuclei or to the meiotic chromosomes utilized anti-MEIG1 and FITC-conjugated goat anti-rabbit IgG antibodies as primary and secondary antibodies, respectively. When cells from testes of pn d8 were analyzed, two distinct populations could be obtained: the 2D cells—mainly testicular somatic cells with few spermatogonia cells, all of which are at the G1 stage of the cell cycle—and the 4D cells—mainly mitotically dividing spermatogonia cells. As can be seen in Fig. 4a, no signal could be detected in either case, confirming that MEIG1 does not localize to the nucleus of the testicular somatic cells and suggesting that it is not attached to the chromatin of spermatogonia cells as well. At pn d10, the 2D population is similar to that of pn d8, whereas the 4D population contains, in addition to the mitotically dividing spermatogonia cells, leptotene spermatocytes that have just entered the meiotic process (Bellve *et al.*, 1977a). *In situ* analysis revealed that while no signal could be obtained in the 2D nuclei, an apparent signal was evident in some of the 4D nuclei or chromosomes (Fig. 4b). This suggests that MEIG1 attaches to the meiotic chromosomes as soon as the leptotene stage when the cells initiate meiosis. A similar pattern of signaling characterized the 4D nuclei or chromosomes from pn d12 except that the number of positively stained chromosome sets increased (not shown). Meiotic chromosomes from FACS-sorted primary spermatocytes at the pachytene stage of prophase I (4D cells from pn d17 testes) exhibited an apparent signal distributed as foci (dots) along numerous meiotic chromosome sets (Fig. 4c). When testicular cells from an adult male were analyzed, four populations were obtained, as expected. The 2D and the 4D cell populations represent testicular somatic and premeiotic cells and the primary spermatocytes, respectively, while the 1D(r) population represents the haploid round spermatids and the 1D(e) population represents the elongated spermatozoa (Malkov *et al.*, 1998). *In situ* analysis of the 1D(r) nuclei revealed an intense signal (Fig. 4d), confirming that MEIG1 remains attached to the chromatin after meiosis has been completed. No signal could be obtained in control experiments executed with secondary antibody only, with preimmune serum, or with anti-MEIG1 antibodies preadsorbed with recombinant MEIG1 prior to use (Figs. 4b–4d). Likewise, no MEIG1 protein could be detected in nuclei or bound to mitotic chromosomes of various somatic cell lines such as the 2B4 lymphocyte cell line (Fig. 5). These results support the notion that MEIG1 indeed interacts directly and specifically with the meiotic chromatin.

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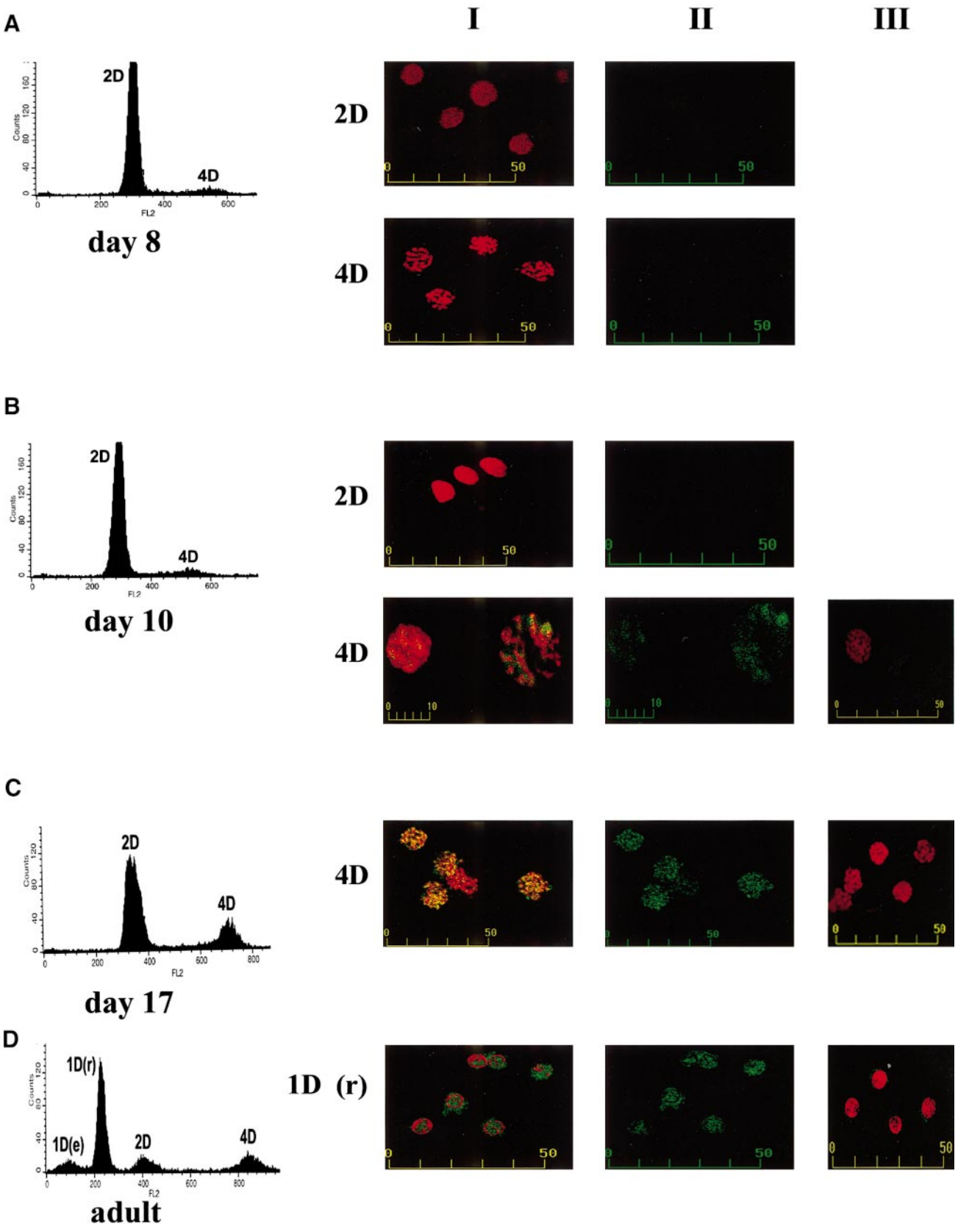
FITC-conjugated goat anti-rabbit IgG antibodies. Nuclei were stained with propidium iodide (PI). Each part is divided into three images: I—double staining of nuclei (red fluorescence) and MEIG1 localization (green fluorescence) with the two images superimposed; II—MEIG1 localization only; III—control double staining with PI and anti-MEIG1 antibodies, preadsorbed with recombinant MEIG1 prior to use, with the two images superimposed. (a) Nuclear and cytoplasmic localization of MEIG1 in a primary spermatocyte (ps), most likely a pachytene spermatocyte, and strictly cytoplasmic localization in what seems to be a testicular somatic cell (sc) in postnatal day 17 testes. (b) Nuclear and cytoplasmic localization of MEIG1 in round spermatids in postnatal day 21–22 testes. (c) Nuclear localization of MEIG1 in spermatozoa (sz) in postnatal day 30 testes. (d) Strictly cytoplasmic localization of MEIG1 in FACS-purified 2D cells (mixture of premeiotic spermatogonia and testicular somatic cells) from normal mature mice. Scale bar indicates size in micrometers.



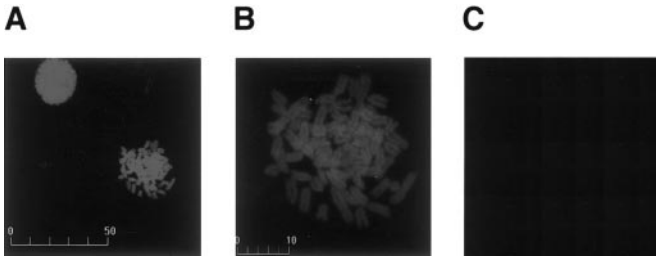



**FIG. 5.** Nuclei and mitotic chromosome preparation from the 2B4 lymphocyte cell line. (a) Double staining with propidium iodide (red fluorescence) and MEIG1 localization (green fluorescence). The two images are superimposed. (b) Higher magnification of the mitotic chromosomes shown in a. (c) MEIG1 localization to the mitotic chromosomes shown in b. Scale bar indicates size in micrometers.

## DISCUSSION

In a previous study (Chen-Moses *et al.,* 1997) we showed that in the testis, MEIG1 appears in multiple phosphorylated forms, including several monomeric forms and two dimeric forms of $M_r$ 31000 and 32000. We also showed that there is a developmentally regulated switch in the relative abundance of the two dimeric forms such that the $M_r$ 31000, which was found to be tyrosine phosphorylated, becomes the dominant form as cells from the first spermatogenic wave enter and progress through meiosis. This suggested that the $M_r$ 31000 form of MEIG1 might play a role during meiosis. In this study, we show that the $M_r$ 31000 dimeric form of MEIG1 appears in the nuclear fraction of testicular protein extracts, whereas the $M_r$ 32000 dimeric form and the monomeric forms of MEIG1 are cytoplasmic. Furthermore, as this nuclear form could not be detected in nuclear extracts of postnatal day 7–8 testes (containing premeiotic spermatogonia and testicular somatic cells) but became apparent in nuclear extracts of testes containing primary spermatocytes at prophase I, we conclude that the nuclear localization is characteristic specifically to the germ cells once they have entered the meiotic process. This conclusion is further supported by the immunocytochemical localization of MEIG1 and the chromosomal *in situ* experiments. No MEIG1 signal could be detected in nuclei of FACS-sorted 2D cells (i.e., somatic or spermatogonia at G1) at any developmental stage or attached to mitotic chromosomes of premeiotic spermatogonia or of other somatic cells (Figs. 3–5). On the other hand, localization to nuclei and attachment of MEIG1 to chromosomes of primary spermatocytes that have just initiated meiosis, in a pattern that correlated with the increased dominance of the $M_r$ 31000, was evident (Figs. 3 and 4). These results suggest that in germ cells, the $M_r$ 31000 dimeric form enters the nucleus during early stages of the first meiotic prophase and binds to the meiotic chromatin. The kinetics of the nuclear localization and chromatin attachment of MEIG1, as shown in this study, together with the previously reported expression of the germ cell-specific transcript of *Meig1* in embryonic ovaries, in which comparable meiotic prophase I stages occur (Don *et al.,* 1994), strongly suggest that MEIG1 plays a role during meiosis which seems not to be restricted to spermatogenesis. We cannot, however, rule out the possibility that MEIG1 associates with the meiotic chromatin in preparation for postmeiotic processes since this association persists through the changes that occur in the components of the chromatin as cells differentiate to spermatozoa. Moreover, it should be emphasized that MEIG1 might play other, non-meiotically related, roles as evident by its ubiquitous cytoplasmic expression in somatic cells.

The focal association of MEIG1 with the meiotic chromosomes is reminiscent of recently reported association patterns of several other proteins with meiotic chromatin. Foci of DNA polymerase $\beta$ have been immunolocalized along synapsed zygotene and early pachytene chromosomes. The enzyme has been implicated in DNA repair and/or semiconservative DNA replication of small regions of the genome that are not replicated during the premeiotic S phase, but rather are completely replicated during zygotene and pachytene (Stern and Hotta, 1987; Plug *et al.,*

**FIG. 4.** Immunological *in situ* localization of MEIG1 in nuclei and chromosome preparations from specific testicular populations obtained from testes of pups at different developmental stages. Testicular cells from each postnatal developmental stage were analyzed by the FACS. The different populations were purified by sorting and were used for nuclei and chromosome preparations. Each part is divided into three images: I—double staining of nuclei (red fluorescence) and MEIG1 localization (green fluorescence) with the two images superimposed; II—MEIG1 localization only; III—control double staining with PI and anti-MEIG1 antibodies, preadsorbed with recombinant MEIG1 prior to use, with the two images superimposed. The control image is presented only when image I is positive for MEIG1. (a) Analysis of cells from postnatal day 8. The 2D cells represent premeiotic spermatogonia and testicular somatic cells, all at the G1 stage of the cell cycle. The 4D cells represent premeiotic spermatogonia cells at the G2 or M stage of the cell cycle. (b) Analysis of cells from postnatal day 10. The 2D cells represent premeiotic spermatogonia and testicular somatic cells, all at the G1 stage of the cell cycle. The 4D cells represent primary spermatocytes at the leptotene stage of prophase I. (c) Analysis of cells from postnatal day 17. The 4D cells represent primary spermatocytes at the pachytene stage of prophase I. (d) Analysis of cells from an adult testis. The 1D(r) cells represent postmeiotic haploid round spermatids. Scale bar indicates size in micrometers.

1997). The ATM, ATR, CHK1 homolog, and c-ABL proteins, all of which are associated with protein kinase activity, have also been detected along zygotene and pachytene meiotic chromosomes (Keegan *et al.*, 1996; Flaggs *et al.*, 1997; Kharbanda *et al.*, 1998). It has been suggested that ATM and ATR might be involved with a meiotic checkpoint pathway, as detectors of DNA damage or incorrect DNA structure. These factors stimulate a phosphorylation cascade that leads to cell cycle arrest until chromosome pairing or genetic recombination are completed (Keegan *et al.*, 1996; Barlow *et al.*, 1998). The CHK1 homolog, whose association with meiotic chromosomes depends upon functional ATM, and c-ABL, whose tyrosine kinase activity is induced at least in part by ATM, are potential transmitters of the information in the checkpoint pathway (Flaggs *et al.*, 1997; Kharbanda *et al.*, 1998). An intriguing speculation would be that MEIG1 might also be involved with such a checkpoint pathway. The kinetics of its nuclear localization and its punctate association with the meiotic chromosomes are in accordance with this possibility. Moreover, MEIG1 contains many potential phosphoacceptor amino acids (Don and Wolgemuth, 1992), making it a potential substrate of the checkpoint kinases. Of special interest is the possibility that MEIG1 is a substrate of c-ABL since the $M_r$ 31000 dimeric form of MEIG1 is specifically tyrosine phosphorylated (Chen-Moses *et al.*, 1997).

A major reservation, however, regarding the potential role of MEIG1 in such a checkpoint pathway is the fact that its nuclear localization persists beyond prophase I, up to the stage of elongated spermatozoa (this study). This pattern might suggest a more structural role that involves chromatin organization in meiosis and thereafter. In this respect, MEIG1 can be suggested as a potential substrate of the NEK2 kinase. NEK2 is a nuclear protein kinase that is abundantly expressed and active in primary spermatocytes, and it has been immunolocalized to meiotic prophase I chromosomes in a punctate pattern. Moreover, NEK2 has been suggested to function in chromosomal organization and/or condensation (Rhee and Wolgemuth, 1997). Whether MEIG1 is indeed a substrate of one of the above-mentioned nuclear kinases or not, it seems to play a role, either regulatory or structural, in the nuclear events occurring during meiosis and thereafter. Assigning MEIG1 as a substrate to specific kinases might clarify the meiotic processes in which it is involved.

An unanswered question is how MEIG1 enters the nucleus, since it does not contain an established nuclear localization signal (NLS). One possibility is that, as a small protein, it simply diffuses into the nucleus through the nuclear pores, as has been shown for several proteins smaller than 45 kDa (reviewed in Silver, 1991; Dingwall and Laskey, 1992). However, this seems unlikely since it does not explain the specific localization of the dimeric form over the monomeric forms, which are smaller, nor the temporal regulation of nuclear association. Since many NLSs are already known, and the list is constantly growing (Boulikas, 1994), it is possible that MEIG1 contains an NLS that has not yet been recognized. The very basic nature of MEIG1 might support this possibility. Another possibility is that MEIG1 interacts with another NLS-containing protein(s) that facilitates its nuclear localization. Yet another mode of nuclear transport of a protein has recently been reported for the meiotic regulator Mei2p in fission yeast. This RNA binding protein binds polyadenylated RNA, mei2RNA, that facilitates its nuclear localization. In the absence of mei2RNA, Mei2p remains in the cytoplasm and meiosis I is blocked unless a nuclear localization signal is artificially added to Mei2p (Yamashita *et al.*, 1998). This might have relevance for MEIG1 since it can also bind RNA efficiently (data not shown).

Phosphorylation and/or dephosphorylation, which can interfere with electrostatic recognition between NLS-containing proteins or complexes and the nuclear pore, are also important factors in the nuclear localization machinery (Jans, 1995). For example, serine dephosphorylation is required for the nuclear import of lamin B2 (Hennekes *et al.*, 1993) and v-Jun (Tagawa *et al.*, 1995), whereas serine phosphorylation activates nuclear localization of c-rel and NF-κB (reviewed in Jans, 1995). Whether MEIG1 has its own NLS or whether it may interact with NLS-containing proteins or other molecular vehicles (such as RNA), the fact that only the $M_r$ 31000 tyrosine-phosphorylated dimeric form is localized to the nucleus and not the serine-phosphorylated $M_r$ 32000 form might suggest that tyrosine phosphorylation and/or serine dephosphorylation reactions play an important role in its nuclear localization.

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